

1: Enzyme Microb Technol 2000 Oct 1;27(7):492-501

Cloning and expression of the nitrile hydratase and amidase genes from *Bacillus* sp. BR449 into *Escherichia coli*.

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A moderate thermophile, *Bacillus* sp. BR449 was previously shown to exhibit a high level of nitrile hydratase (NHase) activity when growing on high levels of acrylonitrile at 55 degrees C. In this report, we describe the cloning of a 6.1 kb *Sal*I DNA fragment encoding the NHase gene cluster of BR449 into *Escherichia coli*. Nucleotide sequencing revealed six ORFs encoding (in order), two unidentified putative proteins, amidase, NHase beta- and alpha-subunits and a small putative protein of 101 amino acids designated P12K. Spacings and orientation of the coding regions as well as their gene expression in *E. coli* suggest that the beta-subunit, alpha-subunit, and P12K genes are co-transcribed. Analysis of deduced amino acid sequences indicate that the amidase (348 aa, MW 38.6 kDa) belongs to the nitrilase-related aliphatic amidase family, and that the NHase beta- (229 aa, MW 26.5 kDa) and alpha- (214 aa, MW 24.5 kDa) subunits comprise a cobalt-containing member of the NHase family, which includes *Rhodococcus rhodochrous* J1 and *Pseudomonas putida* 5B NHases. The amidase/NHase gene cluster differs both in arrangement and composition from those described for other NHase-producing strains. When expressed in *Escherichia coli* DH5alpha, the subcloned NHase genes produced significant levels of active NHase enzyme when cobalt ion was added either to the culture medium or cell extracts. Presence of the P12K gene and addition of amide compounds as inducers were not required for this expression.

PMID: 10978771 [PubMed - as supplied by publisher]

2: Biochemistry 2000 Feb 1;39(4):800-9

Novel heme-containing lyase, phenylacetaldoxime dehydratase from *Bacillus* sp. strain OxB-1: purification, characterization, and molecular cloning of the gene.

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A novel dehydratase that catalyzes the stoichiometric dehydration of Z-phenylacetaldoxime to phenylacetonitrile has been purified 483-fold to homogeneity from a cell-free extract of *Bacillus* sp. strain OxB-1 isolated from soil. It has a M(r) of about 40 000 and is composed of a single polypeptide chain with a loosely bound protoheme IX. The enzyme is inactive unless FMN is added to the assay, but low activity is also observed when sulfite replaces FMN. The activity in the presence of FMN is enhanced 5-fold under anaerobic conditions compared to the activity measured in air. The enzyme has maximum activity at pH 7.0 and 30 degrees C, and it is stable at up to 45 degrees C at around neutral pH. The aerobically measured activity in the presence of FMN is also enhanced by Fe(2+), Sn(2+), SO(3)(2)(-), and NaN(3). Metal-chelating reagents, carbonyl reagents, electron donors, and ferri- and ferrocyanides strongly inhibit the enzyme with K(i) values in the micromolar range. The enzyme is active with arylalkylalldoximes and to a lesser extent with alkylalldoximes. The enzyme prefers the Z-form of phenylacetaldoxime over its E-isomer. On the basis of its substrate specificity, the enzyme has been tentatively named phenylacetaldoxime dehydratase. The gene coding for the enzyme was cloned into plasmid pUC18, and a 1053 base-pair open reading frame that codes for 351 amino

acid residues was identified as the oxd gene. A nitrilase, which participates in aldoxime metabolism in the organism, was found to be coded by the region just upstream from the oxd gene. In addition an open reading frame (orf2), whose gene product is similar to bacterial regulatory (DNA-binding) proteins, was found just upstream from the coding region of the nitrilase. These findings provide genetic evidence for a novel gene cluster that is responsible for aldoxime metabolism in this microorganism.

PMID: 10651646 [PubMed - indexed for MEDLINE]

3: Extremophiles 1999 Nov;3(4):283-91

Characterization of an inducible nitrilase from a thermophilic bacillus.

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Nitrilase activity was induced in the thermophilic bacterium *Bacillus pallidus* strain Dac521 by growth on benzonitrile-supplemented minimal medium. The enzyme had a subunit relative molecular mass of 41 kDa but was purified as a complex with a putative GroEL protein (total M(r), 600 kDa). The enzyme catalyzed the hydrolysis of aliphatic, aromatic, and heterocyclic nitriles with widely varying k_{cat}/K_M values, primarily the result of differences in substrate affinity. Of the nitriles tested, 4-cyanopyridine was hydrolyzed at the fastest rate. Substitution of benzonitrile at the meta or para position either had no effect on catalytic rate or enhanced k_{cat} , while orthosubstitution was strongly inhibitory, probably because of steric hindrance. The effect of catalytic inhibitors was consistent with the presence of active site thiol residues although activity was little affected by putative thiol reagents such as iodoacetate, iodoacetamide, and N-methylmaleimide. Enzymatic activity was constant between pH 6 and 9 with an optimum at pH 7.6. The optimal temperature for activity was 65 degrees C with rapid activity loss at higher temperatures. The purified nitrilase-GroEL complex had the following half-lives of activity: 8.4 h at 50 degrees C, 2.5 h at 60 degrees C, 13 min at 70 degrees C, and less than 3 min at 80 degrees C.

PMID: 10591020 [PubMed - indexed for MEDLINE]

4: Biochim Biophys Acta 1998 Jan 15;1382(1):1-4

Investigation of the potential active site of a cyanide dihydratase using site-directed mutagenesis.

Watanabe A, Yano K, Ikebukuro K, Karube I.

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Cyanide dihydratase has conserved residues in the amino acid sequence with nitrilase, and cyanide hydratase. The conserved amino acid residues in the cyanide dihydratase from *Pseudomonas stutzeri* AK61 were altered by site-directed mutagenesis. The enzyme completely lost its activity of the cyanide hydrolysis by the replacement of cysteine-163 to serine. The replacement of tyrosine-53 to phenylalanine caused an increase of the K_m value of the enzyme for cyanide. Substitution of nine other residues seemed to affect the structure of the enzyme.

PMID: 9507047 [PubMed - indexed for MEDLINE]

5: J Chromatogr B Biomed Appl 1996 May 31;681(1):191-5

High-performance liquid chromatographic study of the aromatic nitrile metabolism in soil bacteria.

Gabriel J, Vekova J, Vosahlo J.

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Simultaneous HPLC determination of bromoxynil, ioxynil and dichlobenil, three aryl nitrile herbicides, and their metabolic products in soil extracts and microbiological media is described. Limits of detection (LODs) ranged from 0.56 to 3.97 ppb. Slight modification of the mobile phase composition allowed determination of 13 other aromatic nitriles. Assay of aromatic nitrile hydratase, amidase or nitrilase activities is possible by the method developed.

PMID: 8798929 [PubMed - indexed for MEDLINE]

6: Plant J 1996 May;9(5):683-91

Transgenic tobacco plants expressing the Arabidopsis thaliana nitrilase II enzyme.

Schmidt RC, Muller A, Hain R, Bartling D, Weiler EW.

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Nitrilase (E.C. 3.5.5.1) cloned from Arabidopsis thaliana converts indole-3-acetonitrile to the plant growth hormone, indole-3-acetic acid in vitro. To probe the capacity of this enzyme under physiological conditions in vivo, the cDNA PM255, encoding nitrilase II, was stably integrated into the genome of Nicotiana tabacum by direct protoplast transformation under the control of the CaMV-35S promoter. The regenerated plants appeared phenotypically normal. Nitrilase II was expressed, based on the occurrence of its mRNA and polypeptide. The enzyme was catalytically active, when extracted from leaf tissue of transgenic plants (specific activity: 25 fkat mg⁻¹ protein with indole-3-acetonitrile as substrate). This level of activity was lower than that found in A. thaliana, and this was deemed essential for the in vivo analysis. Leaf tissue from the transgenic plants converted 1-[13C]-indole-3-acetonitrile to 1-[13C]-indole-3-acetic acid in vivo as determined by HPLC/GC-MS analysis. Untransformed tobacco was unable to catalyze this reaction. When transgenic seeds were grown on medium in the absence of indole-3-acetonitrile, germination and seedling growth appeared normal. In the presence of micromolar levels of exogenous indole-3-acetonitrile, a strong auxin-overproducing phenotype developed resulting in increased lateral root formation (at 10 µM indole-3-acetonitrile). Collectively, these data prove the ability of nitrilase II to convert low micromolar levels of indole-3-acetonitrile to indole-3-acetic acid in vivo, even when expressed at subphysiological levels thereby conferring a high-auxin phenotype upon transgenic plants. Thus, the A1 thaliana nitrilase activity, which exceeds that of the transgenic plants, would be sufficient to meet the requirements for auxin biosynthesis in vivo.

PMID: 8653117 [PubMed - indexed for MEDLINE]

7: Protein Sci 1994 Aug;3(8):1344-6

A new family of carbon-nitrogen hydrolases.

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Using computer methods for database search and multiple alignment, statistically significant sequence similarities were identified between several nitrilases with distinct substrate specificity, cyanide hydratases, aliphatic amidases, beta-alanine synthase, and a few other proteins with unknown molecular function. All these proteins appear to be involved in the reduction of organic nitrogen compounds and ammonia production. Sequence conservation over the entire length, as well as the similarity in the reactions catalyzed by the known enzymes in this family, points to a common catalytic mechanism. The new family of enzymes is characterized by several conserved motifs, one of which contains an invariant cysteine that is part of the catalytic site in nitrilases. Another highly conserved motif includes an invariant glutamic acid that might also be involved in catalysis.

PMID: 7987228 [PubMed - indexed for MEDLINE]

8: Bioorg Med Chem 1994 Jul;2(7):715-21

Enzyme-catalysed enantioselective hydrolysis of racemic naproxen nitrile.

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The bacterial strain *Rhodococcus butanica* (ATCC 21197), which exhibits nitrilase and nitrile hydratase/amidase activities, catalyses the enantioselective hydrolysis of racemic naproxen nitrile (R/S)-1 to furnish a moderate enantiomeric excess of (S)-naproxen (S)-3. Racemic naproxen amide (R/S)-2 is not a good substrate for this strain. Resting cells of the newly selected bacterial strain *Rhodococcus* sp. C3II catalyse the enantioselective hydrolyses of racemic naproxen nitrile (R/S)-1 and naproxen amide (R/S)-2 as well, to give (S)-3 in excellent optical (99% e.e.) and good chemical yields in aqueous medium and in the biphasic system of phosphate buffer/hexane.

PMID: 7858980 [PubMed - indexed for MEDLINE]

9: Proc Natl Acad Sci U S A 1993 Jan 1;90(1):247-51

Nitrilase in biosynthesis of the plant hormone indole-3-acetic acid from indole-3-acetonitrile: cloning of the *Alcaligenes* gene and site-directed mutagenesis of cysteine residues.

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Indole-3-acetic acid is the major auxin in most plants. In Cruciferae, including Brassicaceae, indole-3-acetic acid is synthesized from indole-3-acetonitrile by nitrilase, after indole-3-acetonitrile is formed from tryptophan via indole-3-acetaldoxime or indole glycosinolates as the intermediate. We cloned and sequenced the gene for nitrilase (EC 3.5.5.1), which catalyzes the hydrolysis of indole-3-acetonitrile to indole-3-acetic acid, from *Alcaligenes faecalis* JM3. The amino acid sequence deduced from the nucleotide sequence of the nitrilase gene shows 34.7% identity with that of *Klebsiella ozaenae*

nitrilase. A DNA clone containing the nitrilase gene expressed the active enzyme in *Escherichia coli* with excellent yield. Among five cysteine residues (Cys-40, Cys-115, Cys-162, Cys-163, and Cys-218) in the *Alcaligenes nitrilase*, only Cys-163 was conserved at the corresponding position in the *Klebsiella nitrilase*. Two mutant enzymes, in which Cys-162 and Cys-163 were replaced with Asn and Ala, respectively, were constructed by site-directed mutagenesis. A 35% increase of the specific activity and a large reduction of the K_m for thiophene-2-acetonitrile (which was used as a standard substrate for the nitrilase) were observed in the Cys-162-->Asn mutant enzyme. The Cys-163-->Ala mutation resulted in complete loss of nitrilase activity, clearly indicating that Cys-163 is crucial for the activity and Cys-162 could not provide the catalytic function of Cys-163.

PMID: 8419930 [PubMed - indexed for MEDLINE]

10: Biochem Biophys Res Commun 1992 Sep 16;187(2):1048-54

Cloning and properties of a cyanide hydratase gene from the phytopathogenic fungus *Gloeocercospora sorghi*.

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The Cht gene encoding cyanide hydratase (CHT, EC 4.2.1.66), which detoxifies HCN and is thought to be important in fungal infection of cyanogenic plants, has been cloned from the phytopathogenic fungus *Gloeocercospora sorghi*. The gene was isolated by screening an expression library of *G. sorghi* using a CHT-specific antibody and using one of the positive cDNA clones as a probe in Southern hybridization to identify a 3.1 kb PstI genomic fragment. This PstI fragment expressed CHT activity when transformed into *Aspergillus nidulans*, a fungus that normally lacks CHT activity. Sequence analysis identified a single open reading frame of 1,107 base pairs which encodes a polypeptide of 40,904 daltons. The deduced amino acid sequence of CHT shares 36.5% identity to a nitrilase from the bacterium *Klebsiella pneumoniae* subsp. *ozaenae*.

PMID: 1382413 [PubMed - indexed for MEDLINE]

11: Appl Environ Microbiol 1991 Oct;57(10):3028-32

Production of R-(-)-mandelic acid from mandelonitrile by *Alcaligenes faecalis* ATCC 8750.

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R-(-)-Mandelic acid was produced from racemic mandelonitrile by *Alcaligenes faecalis* ATCC 8750. Ammonium acetate or L-glutamic acid as the carbon source and n-butyronitrile as the inducer in the culture medium were effective for bacterial growth and the induction of R-(-)-mandelic acid-producing activity. The R-(-)-mandelic acid formed from mandelonitrile by resting cells was present in a 100% enantiomeric excess. *A. faecalis* ATCC 8750 has an R-enantioselective nitrilase for mandelonitrile and an amidase for mandelamide. As R-(-)-mandelic acid was produced from racemic mandelonitrile in a yield of 91%, whereas no S-mandelonitrile was left, the S-mandelonitrile remaining in the reaction is spontaneously racemized because of the chemical equilibrium and is used as the substrate. Consequently, almost all the mandelonitrile is consumed and converted

to R-(-)-mandelic acid. R-(-)-Mandelic acid was also produced when benzaldehyde plus HCN was used as the substrate.

PMID: 1660699 [PubMed - indexed for MEDLINE]

12: Agric Biol Chem 1991 Jun;55(6):1459-66

Purification and characterization of nitrilase responsible for the enantioselective hydrolysis from *Acinetobacter* sp. AK 226.

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A nitrilase was purified, apparently to homogeneity, from a cell extract of *Acinetobacter* sp. AK 226, which converts racemic 2-(4'-isobutylphenyl)propionitrile (Ibu-CN) to S-(+)-2-(4'-isobutyl-phenyl)propionic acid (S-(+)-ibuprofen). The molecular weight of the native enzyme was estimated as 580,000 upon gel filtration. The nitrilase hydrolyzed many kinds of nitrile compounds such as aliphatic, aromatic, and heterocyclic mononitriles or dinitriles. The amino-terminal amino acids were sequenced and found to be partly homologous to a nitrilase from *Klebsiella pneumoniae* subsp. *ozanae*. The purified enzyme had a pH optimum of 8.0 and a temperature optimum of 50 degrees C. The enzyme was not affected by chelating reagents, carbonyl reagents, reductants, most metal ions, or thiol reagents except silver ion, p-chloromercuribenzoate, and phenylmercuribenzoate. The reaction with racemic Ibu-CN resulted in the preferential production of S-(+)-ibuprofen, demonstrating that the nitrilase is highly enantioselective to S-(-)-Ibu-CN. In fact, the enzyme showed a 180-fold higher activity for racemic Ibu-CN than that for R-(+)-Ibu-CN.

PMID: 1369128 [PubMed - indexed for MEDLINE]

13: Eur J Biochem 1990 Dec 27;194(3):765-72

A novel nitrilase, arylacetone nitrilase, of *Alcaligenes faecalis* JM3. Purification and characterization.

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A new type of nitrilase, arylacetone nitrilase, has been purified from isovaleronitrile-induced cells of *Alcaligenes faecalis* JM3 in four steps. The purity of the enzyme was confirmed by SDS/polyacrylamide gel electrophoresis, ampholyte electrofocusing and double immunodiffusion in agarose. The enzyme has a molecular mass of about 275 kDa and consists of six subunits of identical molecular mass. The purified enzyme exhibits a pH optimum of 7.5 and a temperature optimum of 45 degrees C. The enzyme is specific for arylacetone nitriles such as 2-thiophenacetone nitrile, p-tolylacetone nitrile, p-chlorobenzylcyanide, p-fluorobenzylcyanide and 3-pyridylacetone nitrile. The enzyme stoichiometrically catalyzes the hydrolysis of arylacetone nitrile to arylacetic acid and ammonia, no formation of amide occurring. However, the enzyme does not attack nitrile groups attached to aromatic and heteroaromatic rings, which are hydrolyzed preferably by the nitrilases known previously. The enzyme requires thiol compounds such as dithiothreitol and 2-mercaptoethanol to exhibit its maximum activity.



PMID: 2269298 [PubMed - indexed for MEDLINE]

14: Appl Environ Microbiol 1990 Oct;56(10):3125-9

Production of S-(+)-ibuprofen from a nitrile compound by *Acinetobacter* sp. strain AK226.

Yamamoto K, Ueno Y, Otsubo K, Kawakami K, Komatsu K.

Pharmaceutical Research and Development Department, Asahi Chemical Industry Company Ltd., Miyazaki, Japan.

S-(+)-2-(4'-Isobutylphenyl)propionic acid [S-(+)-ibuprofen] was produced from racemic 2-(4'-isobutylphenyl)propionitrile (Ibu-CN) by an isolated bacterial strain, *Acinetobacter* sp. strain AK226. Ammonium acetate, acetonitrile, or n-butyronitrile as a carbon source in the culture medium was effective for bacterial growth and induction of this activity. The optimum pH of the reaction was around 8.0. S-(+)-Ibuprofen formed from Ibu-CN by resting cells was present in a 95% enantiomeric excess. *Acinetobacter* sp. strain AK226 appeared to possess a nitrilase for Ibu-CN because 2-(4'-isobutylphenyl)propionamide was not detected in the reaction mixture and 2-(4'-isobutylphenyl)propionamide was not hydrolyzed to S-(+)-ibuprofen. Since S-(+)-ibuprofen was preferentially produced while the R enantiomer of Ibu-CN was left almost intact over the time course of the reaction, the putative nitrilase appeared to be highly specific for the S enantiomer of Ibu-CN.

PMID: 2285318 [PubMed - indexed for MEDLINE]

15: J Antibiot (Tokyo) 1990 Oct;43(10):1316-20

Nitrilase-catalyzed production of pyrazinoic acid, an antimycobacterial agent, from cyanopyrazine by resting cells of *Rhodococcus rhodochrous* J1.

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Using resting cells of *Rhodococcus rhodochrous* J1, in which a large amount of nitrilase is induced, a simple and efficient bioconversion process for the production of pyrazinoic acid, an antimycobacterial agent, through catalysis by a nitrilase was developed. The reaction conditions for production of pyrazinoic acid were optimized. Under optimum conditions, 3.5 M cyanopyrazine was converted to pyrazinoic acid, with a molar conversion yield of 100%. The highest yield achieved corresponded to 434 g of pyrazinoic acid per liter of reaction mixture. The synthesized pyrazinoic acid was isolated and identified physico-chemically.

PMID: 2258329 [PubMed - indexed for MEDLINE]

16: C R Acad Sci Hebd Seances Acad Sci D 1976 Sep 20;283(5):571-3

[Nitrilase activity in several bacteria].

[Article in French]

Arnaud A, Galzy P, Jallageas JC.

Eighteen strains of Bacteria from the genus Bacillus, Bacteridium, Micrococcus and Brevibacterium were isolated. They have a very general nitrilase activity that acts on all the substrates with nitrile function.

PMID: 825308 [PubMed - indexed for MEDLINE]

17: Folia Microbiol (Praha) 1976;21(3):178-84

Amidase activity of some bacteria.

Arnaud A, Galzy P, Jallageas JC.

The amidase activity of bacteria possessing a high nitrilase activity was found to display the same spectrum although the bacteria may belong to different taxonomic groups, Bacillus, Bacteridium, Micrococcus, Brevibacterium. The spectrum of amidase activity, although very broad, is more restricted than that of nitrilase activity. Internal amides as well as vinyl-bound amides are not hydrolyzed.

PMID: 947836 [PubMed - indexed for MEDLINE]

18: Biochem Soc Trans 1976;4(3):502-4

Purification and properties of an unusual nitrilase from Nocardia N.C.I.B. 11216.

Harper DB.

PMID: 12045 [PubMed - indexed for MEDLINE]